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Apoptosis of hair follicle cells in the second-degree burn wound unders hypernatremic conditions

T. Harada[a]*, S. Izaki[b], H. Tsutsumi[a], M. Kobayashi[a], K. Kitamura[b]

[a]*Department of Emergency and Critical Care Medicine, Saitama Medical Centre, Saitama Medical School, Kamoda 1981, Kawagoe, Saitama 350, Japan*

[b]*Department of Dermatology, Saitama Medical Centre, Saitama Medical School, Kamoda 1981, Kawagoe, Saitama 350, Japan*

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Abstract

Progressive burn wound necrosis is an important factor as a cause of delayed healing during clinical therapy of burns. Among the causes of progressive necrosis have been attributed an insufficient blood supply or a dehydration at the zone of stasis just beneath the zone of coagulation. In a previous study evidence was presented that hypernatremia, an osmotic injury, may act to promote progressive tissue or cell death of the superficial dermal wound resulting from a heat injury. To test this hypothesis pathological features of cell death in the second-degree burn wound in the rat with hypernatremia were investigated and evidence for apoptosis in hair follicle cells was observed. Rats in the hypernatremic group were administered 10 ml of hypertonic sodium solution (850 meq l^{-1}) and the control rats were treated with 10 ml of hyponatremic solution (100 meq l^{-1}) to prevent hypernatremia. After 24 h postburn the average incidence of hair follicles (ratio to the normal skin) in the hypernatremic group was 30.1 ± 11.6 per cent and significantly lower when compared with the control group (87.6 ± 6.0 per cent). The numbers of hair follicles were studied by haematoxylin and eosin stain, and the apoptotic process was investigated by an immunochemical assay and electron microscopy.

Keywords: Burns wound; Hair follicle cells; Apoptosis

1. Introduction

All clinicans who care for burned patients recognize the occurrence of progressive tissue necrosis in burn wounds. The progressive aggravation of a burn wound, especially from a superficial dermal wound to a deep wound, is of great concern because resultant clinical outcomes differ considerably. If hair follicles and their cells are well preserved, the burn wound heals spontaneously in 2 weeks and hair is preserved. If not, skin graft operations are frequently needed and hair never regrows. Progressive tissue or cell death of the burn wound is an important influence on the clinical course of burn therapy.

A major pathological feature of this phenomenon is that the zone of coagulation of the burn wound increases in depth over several days after burn injury. The suggested causes of this progressive necrosis include an insufficient blood supply [1] or a dehydration [2] at the zone of stasis just beneath the zone of coagulation. If it is a second-degree burn wound, the zone of stasis involves the hair follicles in the subpapillary dermis which are a source of epithelial cells for skin regeneration. To diagnose the depth of the wound and to predict whether the wound will remain as superficial or convert into a deeper wound, several methods have been advocated. Although these methods have had some success, this problem is not fully resolved [3,4]. Clinical observations of many burned patients, however, suggest that there may be other pathology involved in the natural history of this progression. In order to investigate the mechanisms involved in this pathological change, those patients who suffered from complications occurring after the acute phase, despite coming through the burn shock stage, were reviewed. From these studies it appeared that there is a close relationship between hypernatremia and progressive tissue necrosis in the burn wound (personal observations). It is possible that hypernatremia, an osmotic

*Corresponding author.

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injury, may act as a promoter to provoke progressive tissue or cell death of the superficial dermal wound which has suffered a heat injury as an initiator [5]. To test this hypothesis the pathological features of cell death in the second-degree burn wound of the rat with hypernatremia were investigated and evidence was observed of apoptosis in hair follicle cells. The process may lead to delayed healing of the superficial burn wound, by decreasing the supply of epithelial cells for the healing process.

2. Material and methods

2.1. Animals and tissue preparation

An experimental skin burn model was prepared as described below. Adult male Wistar rats (280–300 g) were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). The skin of the back was shaved and thoroughly depilated with a proprietary depilatory cream. Burn injury was made as a contact burn with a metal plate (70°C, 5 s) on the skin of the back to produce a second-degree burn wound [6]. Three round wounds (1 cm in diameter) were made on both left and right sides symmetrically; in total six wounds in each rat. Five rats in the hypernatremic group were given 10 ml of hypernatremic solution (850 meq l^{-1} of sodium chloride) by intraperitoneal injection to produce hypernatremia. Five rats in the control group were given 10 ml of hyponatremic saline (100 meq l^{-1} of sodium chloride) to prevent the hypernatraemia resulting from increased water loss by evaporation through the burn wound surface. This hyponatraemic solution was regulated to be isotonic with 5 per cent glucose solution in order to avoid intravascular haemolysis. Two specimens of the burned skin which involved subdermal muscle and the adjoining normal skin were harvested from each rat at 4, 8 and 24 h after burn injury under inhalation ether anaesthesia or intraperitoneal administration of sodium pentobarbital (50 mg kg^{-1}). These specimens were preserved for light microscopic study and small samples excised from the tissue samples were treated for electron microscopic observation.

The rats in this study were treated strictly according to the Guidelines for Animal Experimentation set down by the Ethical Committee of Saitama Medical School.

2.2. Light microscopic study

Routine haematoxylin and eosin (H&E) stain was performed. The numbers of hair follicles of both groups were counted in the specimens after 4, 8 and 24 h. The ratio of the number in the wound (1 cm in width) to that in the adjoining normal skin (1 cm in width) was calculated in each specimen. Unpaired Student's *t*-test was used to compare results with the control group.

Terminal deoxydinucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay was performed to confirm an apoptosis using the apoptosis detection kit, ApopTag™ Plus (Oncor, USA). Paraffin sections were dewaxed, rehydrated, treated with digestive enzyme (10 $\mu g\ ml^{-1}$) (Oncor, USA), and blocked for endogenous peroxidase activity with 3 per cent H_2O_2. Subsequent end-labelling with TdT enzyme was carried out for 1 h at 37°C in a humidifying chamber. After end-labelling, sections were washed in phosphate-buffered saline (PBS), and incubated with anti-digoxigenin-peroxidase, rinsed in PBS, and stained with DAB substrate. Apoptotic nuclei were identified as brown-stained cells. In one of the specimens showing apoptosis after 4 h, complete disappearance of the hair follicles after 24 h was detected.

2.3. Electron microscopic study

The same specimen used in the TUNEL assay was investigated. It was immersion-fixed in 1 per cent formaldehyde and 2.5 per cent glutaraldehyde and rinsed with PBS. This tissue was rinsed and stored in PBS containing 5 per cent sucrose, postfixed in 1 per cent aqueous OsO_4, and stained with 0.2 per cent uranyl acetate. After dehydration, embedding in epon, sectioning and staining with uranyl acetate and lead citrate, the tissue was examined with a Hitachi H-600 electron microscope.

2.4. Gel electrophoresis

Evidence of internucleosomal DNA fragmentation and randomized DNA fragmentation in the burned dermis 4 h after injury was sought by gel electrophoresis with a radioactive end-labelling method described previously [7]. Briefly, frozen skin tissue was treated with collagenase and was homogenized in buffer, and incubated at 68°C for 60 min. The precipitates were spun down and the supernatants digested with DNAase-free RNAase at 37°C for 60 min, extracted with phenol/chloroform, precipitated with isopropanol at −20°C overnight, dried, and redissolved in distilled water. After end-labelling by the radioactive substances, the labelled DNA was precipitated. The DNA pellets were dissolved in distilled water, and fractionated using 2 per cent agarose gels. After exposing to an X-ray film the patterns of DNA fragments were investigated.

3. Results

H&E stain study revealed that the zone of coagulation which can be recognized by the degenerative collagen fibres due to the direct effect of a thermal injury ranges from 10 to 20 per cent of the total thickness of the dermis in all the specimens. The residual portion of the dermis except the zone of coagulation is the zone of stasis, which in the early stages has the potential to remain as viable or to progress to necrosis. The results of this study are summarized in Table 1. The serum sodium concentration in the hypernatremic group was 162.8 ± 5.8 meq l^{-1} and in the control group 140.8 ± 2.0 meq l^{-1}, both at 24 h postburn. The hair follicle ratio in the hypernatremic group was 30.1 ± 11.6 (per cent) and in the control group the ratio was 87.6 ± 6.0. Significant differences were recognized at this serum sodium concentration and hair follicle disappearance was observed.

After 4 h postburn the morphological changes of hair follicle cells began (Fig. 1). Nuclei of hair follicle cells begin to shrink. They are removed by phagocytic cells in 24 h, leaving behind the fibres of follicular

Table 1
Results of study of serum sodium concentration and hair follicle ratio[a] (means ± SE, $n = 10$)

	Hours postburn		
	4	8	24
Hypernatremic group			
Serum sodium concentration (meq l^{-1})	171.4 ± 9.3^{b}	168.3 ± 8.9^{b}	162.8 ± 5.8^{b}
Hair follicle ratio (%)	97.1 ± 1.1	76.5 ± 9.5^{b}	30.1 ± 11.6^{b}
Control group			
Serum sodium concentration (meq l^{-1})	141.5 ± 3.7	143.2 ± 3.1	140.8 ± 2.0
Hair follicle ratio (%)	96.2 ± 1.6	95.0 ± 1.4	87.6 ± 6.0

[a]Hair follicle ratio means the ratio of the number of hair follicles in the burned dermis to that in the adjoining normal dermis.
[b]Significant difference compared with the control group using unpaired Student's t-test ($P < 0.01$).

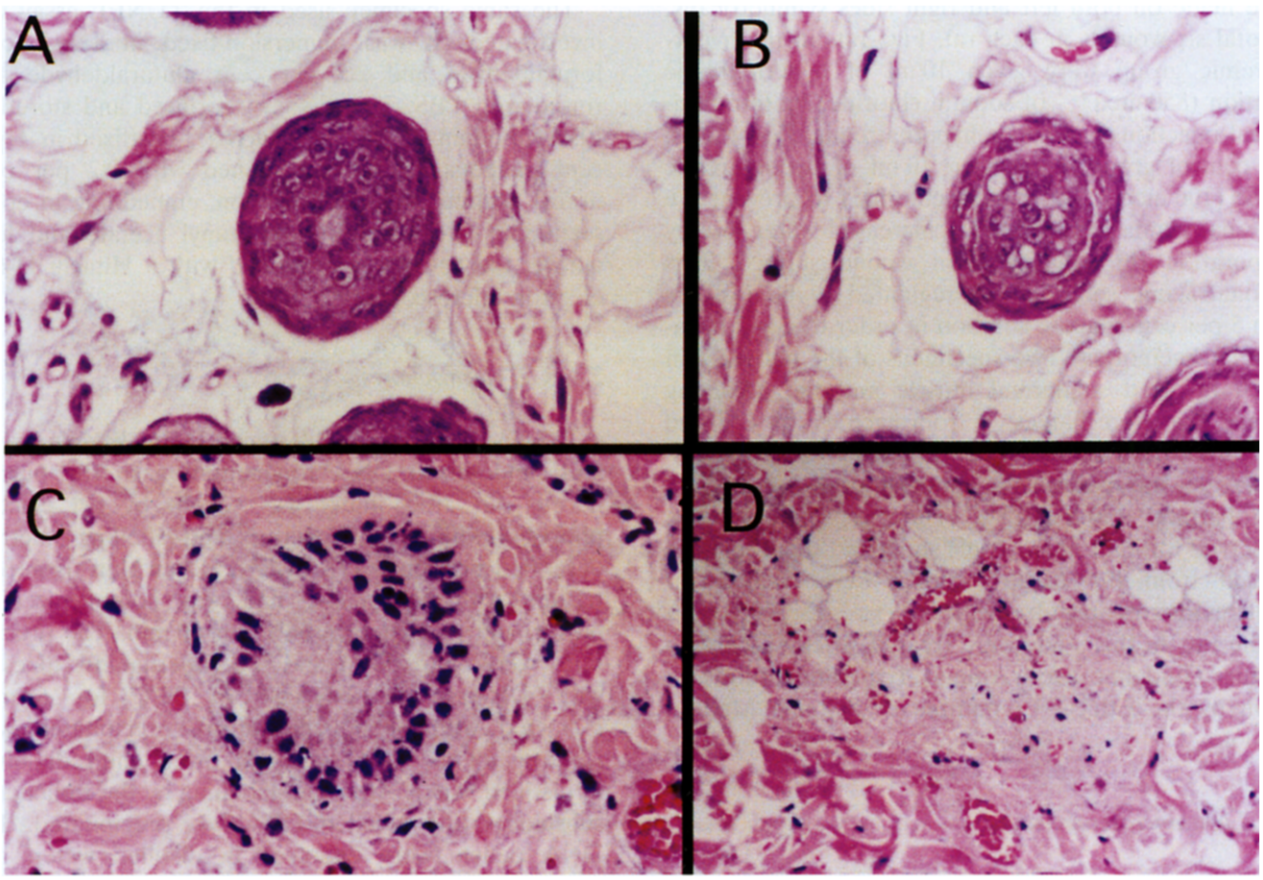

Fig. 1. Haematoxylin and eosin stain. **A**, normal hair follicle cells. **B**, 4 h postburn. Some cells begin to shrink and disappear. **C**, 8 h postburn. This effect seems to extend to all the cells in the structure in a few hours. Nuclei shrank and looked condensed. **D**, 24 h postburn. All the cells have already disappeared, leaving behind the fibres of the follicle matrix. (Original magnitude A–C × 400, Dx200.)

matrix. After 24 h most of follicles have disappeared in the hypernatremic group (Fig. 2). In one of the specimens showing apoptosis after 4 h, complete disappearance of the hair follicles after 24 h was observed. In the rats treated with hyponatremic solution (the control group) most of the hair follicles are well preserved despite the presence of much oedema compared with the rats suffering from hypernatremia.

The TUNEL-positive cells are brown-stained and are characterized by a round and shrunken morphology (Fig. 3). This apoptotic process often begins in the medullary cells of the hair follicles and extends to all the cells.

In the electron microscopic study the specimen of the hypernatremic groups shows condensed chromatin, blebbing of the nuclear membrane and well-preserved mitochondria (Fig. 4). This condensation is due to the internucleosomal DNA fragmentation instead of the normal reticular structure.

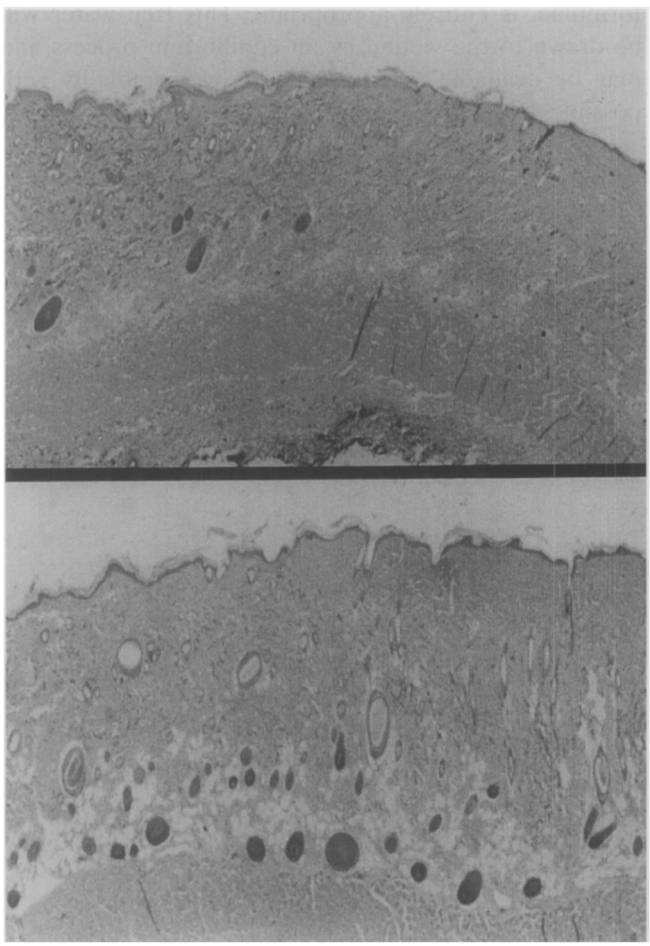

Fig. 2. Haematoxylin and eosin stain. Histological change of the dermis after 24 h. **Upper**, dermis of the rat treated with the hypernatremic solution. The right half is a burned region and the left half is an intact region adjoining the wound. Most of the hair follicles in the burned region have disappeared. **Lower**, dermis of the rat in the control group. Most of the hair follicles are well preserved despite much oedema. (Original magnitude ×40.)

In the gel electrophoresis study random DNA fragmentation in the form of a DNA smear was not seen and no DNA ladder was detectable.

4. Discussion

Recent reports present evidence for apoptosis in hair follicle cells. These reports demonstrate that apoptosis is a feature in the normal homeostatic process [8–13], the developmental process [14], alopecia areata [15,16], patients with HIV infection [17], under treatment with immunosuppressive agents [18], under treatment with a carcinostatic agent [19], following irradiation [20,21], treatment with epidermal growth factor [22], and in bel-2-deficient mice [23]. The mechanism of several types of hair loss are now under investigation by many researchers.

In this experimental study apoptosis in hair follicle cells in the burn wound was shown morphologically by light microscopy (H&E and TUNEL stain) and electron microscopy. On examination of DNA by gel electrophoresis a laddering pattern of DNA fragments could not be shown as the majority of DNA isolated would not be from hair follicles. Further experiments using cultured hair follicle cells without any other components of the dermis would be needed to confirm a laddering pattern, resulting from apoptosis solely in hair follicles.

Why do hair cells in the burned dermis select the pathway to apoptosis? In this experimental study it is suggested that a heat injury would be the initiating factor and an osmotic injury the promoting factor leading to the apoptotic process instead of cell repair [5]. Heat-injured cells produce heat shock proteins which are involved in the repair of intracellular damage [24]. During this phase cells sense information about the intra- and extracellular environment that determines whether they will continue the repair process or select cell death. It appears that different signalling pathways ultimately converge to activate a common apoptotic programme [25]. A decision will be influenced by delicate differences in several factors. Our study suggests that hair follicles disappear in an 'all or nothing' pattern. When the burn injury was more or less severe than that induced in this model, we could not find any difference in behaviour, at least at the stage of pilot studies.

This suggests that hypernatremia is a malign influence on cell viability and survival. The hyperosmotic, especially hypernatremic, condition seriously impairs several cellular functions. Hypernatremia may exhaust intracellular ATP as a result of activation of the Na^+–K^+ pump and this leads to suppression of other energy-dependent functions to a critical degree [26]. Under hypernatremic conditions the heat-damaged

hair follicle cells might be unable to maintain specific energy-dependent functions and accelerate towards apoptosis. A recent report suggests that the relative increase of intracellular oxidative stress reinforces the tendency to apoptosis [27]. The decrease of the intracellular ATP content will also suppress the reduction system dealing with oxidative stress which thus prevents the start of the apoptotic process. Other reports investigate the existence of intercellular factors which act to prevent or induce apoptosis in the regulation of harmonized social controls on cell survival and death [28,29]. In the severely compromised dermis there will be a strong competition for survival among cells. The lost balance of cellular social control may induce abnormal cell death or decrease chances of survival [30]. In this burned model the heat and osmotic injuries may interefere with appropriate activation of the inflammatory response and possibly induce more extended necrosis of the dermis. Further cytological and biochemical studies are needed to investigate the mechanism of these processes.

A more important problem for clinicians is that this rapid aggravation can occur at any time when systemic hypernatremia or, more importantly, a local hypernatremic condition in the burn wound is seen. (Although it is not confirmed in our study, serum hyperosmolality induced by some other osmotically active compounds seems to have less influence on the wound progression.) Hypernatremia sometimes occurs on the first or second day during the initial fluid resuscitation with some hypertonic sodium solutions. It may also occur even in the cases treated with several types of isotonic solutions on the fourth or fifth day at the end of the refilling phase when water loss exceeds sodium wash-out. This is attributed to increased evaporation from the wound surface with or without depressed renal function. At all events some burned patients need more free water than that expected as a medium of exreting the abundant sodium deposited in the body during resuscitation in the shock stage. In relation to wound resuscitation we recommend administration of hyponatremic isotonic solutions containing 5 per cent sugar solutions for the correction of insensible water loss, such as suggested in many historic fluid formulae, is entirely appropriate. This free water will be drawn to the wound by an equilibrium process and may be available to correct local hypersmolality with possible better preservation of cell numbers, viability

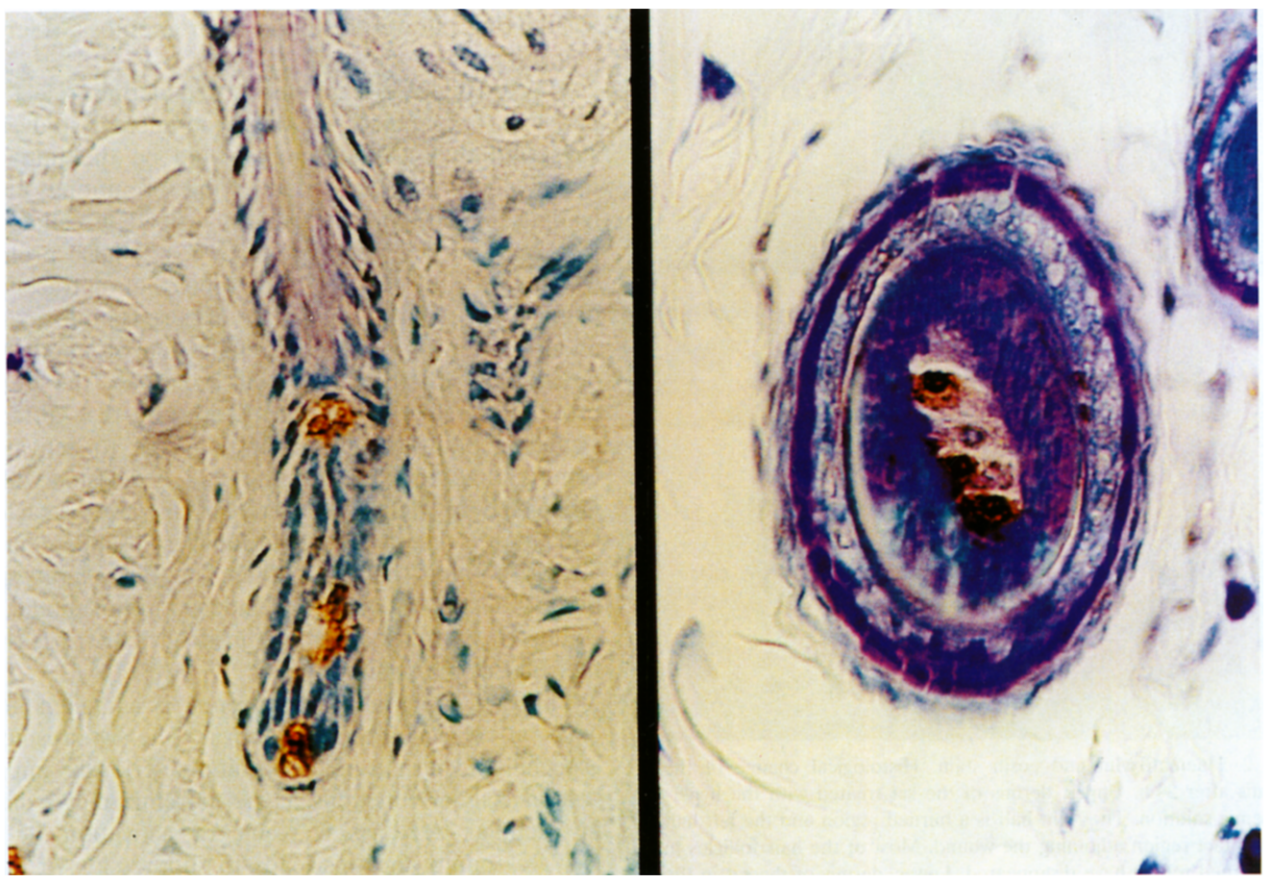

Fig. 3. TUNEL stain. **Left**, apoptotic cells in the hair follicle are recognized as brown-stained cells. (Original magnitude ×100.) **Right**, apoptotic change often begins from the medullary cells of the hair follicles. (Original magnitude ×400.)

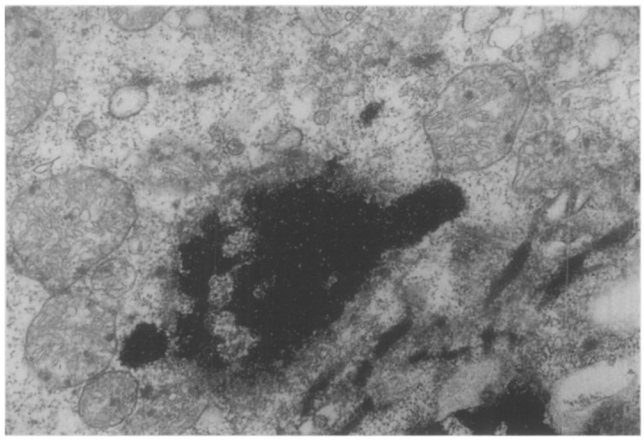

Fig. 4. Ultra structure of an apoptotic cell. This cell shows condensed chromatin, blebbing of the nuclear membrane and well-preserved mitochondria. (Original magnitude ×16,000.)

and tissue integrity. In this way the promotion of continuing viability in the cells of the affected tissue could avoid progressive burn wound necrosis.

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